Research Note

Comparison of Four *Salmonella* Isolation Techniques in Four Different Inoculated Matrices[1,2]

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ABSTRACT The poultry industry is now operating under increased regulatory pressure following the introduction of the pathogen reduction and hazard analysis critical control point (HACCP) rule in 1996. This new operation scheme has greatly increased the need for on-farm food safety risk management of foodborne bacteria, such as *Salmonella*. Information needed to make informed food safety risk management decisions must be obtained from accurate risk assessments, which rely on the sensitivity of the isolation techniques used to identify *Salmonella* in the production environment. Therefore, better characterization of the *Salmonella* isolation and identification techniques is warranted. One new technique, immunomagnetic separation (IMS), may offer a benefit to the poultry industry, as it has been shown to be efficacious in the isolation of *Salmonella* from various sample matrices, including some poultry products. In this work, we compared the isolation ability of 4 *Salmonella*-specific protocols: IMS, tetrathionate (TT) broth, Rappaport-Vassiliadis R10 (RV) broth, and a secondary enrichment (TR) procedure. All 4 methods were compared in 4 different spiked sample matrices: Butterfield's, poultry litter, broiler crops, and carcass rinses. IMS was able to detect *Salmonella* at 3.66, 2.09, 3.06, and 3.97 $\log_{10}$ cfu/mL in Butterfield's, poultry litter, carcass rinse, and broiler crop matrices, respectively. For the broiler litter and Butterfield's solution, there were no ($P > 0.05$) differences among the 4 isolation protocols. However, in the carcass rinse and crop samples, there were no differences among the isolation of *Salmonella* using RV, TR, or TT, but all 3 were ($P \leq 0.05$) more successful at recovering *Salmonella* than the IMS method.

(*Key words*: *Salmonella*, immunomagnetic separation, methodology, secondary enrichment, carcass rinse)

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INTRODUCTION

Over the years, numerous reports have been published comparing various methods for isolating and identifying *Salmonella* from various sample types. Many of these publications have focused on isolation of *Salmonella* from within poultry samples, either meat products or preharvest environmental samples. A current review of the literature suggests that no one method has superiority over another and that the sensitivity and specificity of the methods depends on the sample type as well as the isolation conditions. Most of the studies reviewed have concentrated on comparing various selective enrichment broths, specifically tetrathionate (TT), Rappaport-Vassiliadis (RV), and selenite-cystine (Vassiliadis et al., 1974; Vassiliadis et al., 1976; Vassiliadis et al., 1978; Cox et al., 1982; Davies and Wray, 1994; Read et al., 1994; Hammack et al., 1998; Huang et al., 1999).

Also, when samples are analyzed for the presence or absence of *Salmonella*, the sample matrix composition should be considered when attempting to interpret the results of the analysis (Davies et al., 2000). It has been demonstrated that sample makeup can reduce the sensitivity and specificity of an isolation protocol (Skjerve and Olsvik, 1991).

An understanding of the characteristics of an isolation method used is essential when making production or processing risk-management decisions, such as strategic scheduling (Long et al., 1980; Hargis et al., 2000). These decisions are based on risk assessments, which require accurate results, obtained from sample analysis.

To obtain accurate results from various sample matrices, identification of the most appropriate methodology for microbial evaluation of samples containing low levels

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Abbreviation Key: DB = Dynal Biotech anti-*Salmonella* Dynabeads; DSE = delayed secondary enrichment; IMS = immunomagnetic separation; NA = nalidixic acid; NO = novobiocin; PC = Butterfield's solution; RV = Rappaport-Vassiliadis; TR = secondary enrichment; TT = tetrathionate.

of *Salmonella* is crucial. Additionally, methodologies that provide for rapid screening are essential. New technologies, such as immunomagnetic separation (IMS), may offer an opportunity for detecting *Salmonella* at lower levels in various preharvest sample matrices in less time when compared with traditionally used isolation methods. The use of IMS has been reported in examining raw eggs, where it was shown to be efficient when the egg samples were supplemented with ferrous sulfate to aid the *Salmonella* growth prior to IMS (Cudjoe et al., 1994). It was also shown that the ability of IMS, in combination with flow cytometry (Wang and Slavik, 1999) and with immunooptical absorption (Liu et al., 2001), to isolate *Salmonella* from poultry carcass rinses could detect the pathogen at low levels. However, there are no reports in the literature that indicate the efficacy of IMS in the analysis of preharvest poultry samples, such as litter or crops.

The purpose of this work was to characterize 4 *Salmonella* isolation methods, 2 traditional, 1 IMS, and 1 secondary enrichment method, in matrices where the pathogen is commonly found in the poultry production continuum.

MATERIALS AND METHODS

The 2 traditional *Salmonella* selective isolation broths used were TT[4] and RV (R10).[5] The IMS method used was Dynal Biotech anti-*Salmonella* Dynabeads (DB).[6] For the secondary enrichment method (TR), TT was used as the primary enrichment and RV was used as the secondary enrichment broth. The 4 matrices used were Butterfield's solution (PC), broiler litter, carcass rinses, and crops from market age chickens.

Bacterial Culture

A pure culture of *Salmonella enterica* serotype Typhimurium NN,[7] which is resistant to nalidixic acid (NA) and novobiocin (NO), was used as the test organism. The culture was maintained on brilliant green agar[4] plates supplemented with 25 μg/mL of NO and 20 μg/mL of NA at refrigerated temperatures until needed.

To prepare the inoculum for each sample matrix, an overnight culture of *S.* Typhimurium NN picked from a single isolated colony was grown in sterile brain heart infusion broth[4] containing NO and NA. The broth was incubated at 37°C in an environmental shaker.[8] A 1-mL aliquot of the overnight culture was transferred to 75 mL of brain heart infusion broth containing NN broth, and the optical density was measured at 600 nm.[9] The freshly inoculated culture was incubated at 37°C in the environmental shaker until an optical density at 600 nm of 0.7 was reached, at which point a 10-fold serial dilution (10^0 to 10^{-10} cfu/mL) was made. Enumeration plate counts were performed on the serial dilutions to determine the culture concentration at the time of inoculation.

Sample Inoculation

To compare the 4 isolation protocols (RV, TT, TR, and DB) without interference from confounding factors likely to be present in litter, carcass rinse, and crop sample, 1-mL aliquots of each inoculum dilution (10° to 10^{-10}) were used to inoculate sterile Butterfield's solution (first sample matrix). This procedure was repeated so that a total of 10 replicates were performed using the same bacterial culture.

A second sample matrix was crop samples obtained from a local poultry processing facility. Crops from market age broilers (n = 110) were collected aseptically, placed into sterile WhirlPak bags,[10] transported on wet ice to the laboratory, and stored at 4°C until used.

In the laboratory, 1-mL aliquots from each of the *Salmonella* culture dilutions were used to inoculate 11 crop samples (weighing an average of 8.2 g). Each inoculated sample was mixed vigorously by hand for 30 s. This process was repeated 9 more times to provide a total of 10 replicate sets of crops inoculated with 10^0 to 10^{-10} dilutions of the *Salmonella* culture.

Litter served as the third sample matrix for the inoculation study. A pooled sample was collected from a broiler grow-out house located at Mississippi State University South Farm following harvest of the birds and transported to the laboratory for further processing. The litter was divided into 110 aliquots of 25 g each, which were placed into sterile filtered WhirlPak bags. One-milliliter aliquots of each of the *Salmonella* culture dilutions were used to inoculate 11 bags containing litter, which were mixed vigorously by hand for 30 s. This process was repeated 9 times to provide a total of 10 replicates of litter matrix samples inoculated with 10^0 to 10^{-10} dilutions of the *Salmonella* culture.

The fourth sample matrix, carcass rinse, was obtained from a local poultry processing plant as part of the plant's routine sampling program. The Butterfield's rinse sample was divided into 110 aliquots of 9 mL each and placed into sterile 50-mL conical bottom centrifuge tubes. One-milliliter aliquots of each of the *Salmonella* culture dilutions were used to inoculate 11 rinse tubes. This process was repeated 9 times to provide a total of 10 replicates of carcass rinse matrix samples inoculated with 10^0 to 10^{-10} dilutions of the *Salmonella* culture.

Pre-Enrichment, Selective Enrichment, and Isolation

Nonselective pre-enrichment broth, Butterfield's solution (0.00031 *M* KH_2PO_4, pH 7.2) was added to the litter and crop samples at 1:10 wt/vol to allow microorganisms to recover from injury resulting from sample preparation

[4]Remel Inc., Lenexa, KS.
[5]Difco Laboratories, Detroit, MI.
[6]Neogen Corporation, Lansing, MI.
[7]Obtained from the National Veterinary Services Laboratory, Ames, IA.
[8]Series 25, New Brunswick Scientific, Edison, NJ.
[9]Virian DMS 200 UV visible spectrophotometer, Victoria, Australia.
[10]Nasco, Fort Atkinson, WI.

or from deleterious effects of the environment. Although this step was not necessary for this study, it was included to fully simulate normal practice when dealing with field samples. No additional Butterfield's solution was added to the PC or carcass rinse samples because both sample types already contained the pre-enrichment broth. All samples were incubated overnight at 42°C before being subjected to each of the selective enrichment and isolation protocols.

For the RV and TT protocols, broths were prepared according to the manufacturer's directions. Nine-milliliter aliquots of both broths were aseptically transferred to eleven 50-mL conical bottom centrifuge tubes and inoculated with 1 mL of each sample for each replicate. Tubes were vortexed and incubated at 42°C overnight.

Dynal anti-*Salmonella* Dynabeads (DB) were purchased[6] and then stored at 4°C until used. Following the manufacturer's suggested protocol, 20 μL aliquots of magnetic bead complex were aseptically added to 1.5-mL microfuge tubes. One milliliter of each sample was added to corresponding tubes. Tubes were vortexed and incubated at room temperature for 30 min with intermittent shaking. Tubes were placed into a magnetic particle concentrator[11] and left undisturbed for 10 min to allow the magnetic beads to concentrate onto the side of the tubes. The supernatant was aspirated using sterile Pasteur pipettes, leaving the beads concentrated on the sides of the tubes. One milliliter of sterile PBS-Tween 20 wash solution (0.15 *M* NaCl, 0.01 *M* Na_2HPO_4, pH 7.4, and 0.05% Tween 20) was added to each tube. Tubes were shaken to evenly distribute the beads in the wash solution and allowed to sit undisturbed for 10 min. Samples were washed 2 more times following the same procedure. After the third wash, beads were resuspended in 100 μL of PBS-Tween 20.

For the TR method, the original TT tubes were re-incubated an additional 24 h at 42°C. After re-incubation, 0.1-mL aliquots of each tube were transferred into 9.9 mL of fresh RV and incubated at 42°C for 24 h. This method is a slight modification of a previously published method (Barber et al., 2002).

After incubation, a loop full of the RV, TT, and TR samples and 50 μL of the DB samples were streaked onto xylose-lysine tergitol 4 (XLT4)[4] plates supplemented with 25 μL of NO and 20 μL of NA, followed by overnight incubation at 37°C. The xylose-lysine tergitol 4 plates containing suspect *Salmonella* colonies, which were red with black centers, were further characterized by observing the typical biochemical reactions on triple sugar iron agar and lysine iron agar slants. Isolates producing positive results on the slants were also tested serologically (*Salmonella* O antiserum Poly A-I & Vi[4]).

Statistical Analysis

For each of the 4 sample matrices (PC, litter, carcass rinse, and crop), differences in $\log_{10}$ cfu/mL of *Salmonella* detected among the 4 isolation protocols were evaluated by ANOVA (GLM procedure[12]). Least square means with Tukey's adjustment for multiple comparisons was used to determine the significance of differences among treatment means.

RESULTS

The initial concentration of the inoculum cultures for each of the matrices was 10^8 cfu/mL as determined by enumeration plate counts. For the PC matrix, the TR protocol demonstrated the lowest level of *Salmonella* detection with a mean of 2.56 $\log_{10}$ cfu/mL (Table 1); however, there were no differences among the four isolation protocols evaluated.

When evaluating the 4 isolation protocols in the litter matrix, TR demonstrated the lowest level of detection (1.79 $\log_{10}$ cfu/mL); however, there were no differences among the 4 isolation protocols.

In the crop samples, TR again provided the lowest level of detection (2.07 $\log_{10}$ cfu/mL) compared with the other 3 protocols. There were no differences among the TR, TT, and RV protocols; however, there was a difference ($P < 0.001$) in the isolation ability of the DB protocol when compared with the other 3 methods.

Results in the fourth sample matrix, carcass rinses, were similar to results found for the crop samples. The TR method again provided the lowest level of detection with a mean of 0.76 $\log_{10}$ cfu/mL. There were no differences detected among the TR, TT, and RV protocols; however, there was a difference ($P < 0.0001$) between DB and the other 3 methods.

DISCUSSION

When performing preharvest risk assessment, identification of the most appropriate sites that give the highest probability of isolating *Salmonella* is essential. Previously published work has demonstrated that poultry litter and broiler crops are 2 primary sites positive for *Salmonella* within the production continuum (Fanelli et al., 1971; Snoeyenbos et al., 1982; Corrier et al., 1991, 1999a,b; Hargis et al., 1995; Ramirez et al., 1997; Byrd et al., 2001, 2002). For that reason, these 2 matrices were chosen for study in this work. The carcass rinse samples were used for the inoculation study to simulate an in-plant *Salmonella* monitoring program. The litter was studied because it has been demonstrated, when compared with cloacal sampling and other environmental samples, that litter samples provide a better assessment of the *Salmonella* status of a house preharvest (Olesiuk et al., 1969; Sasipreeyajan et al., 1996).

The DB method did not provide the lowest level of *Salmonella* detection in the 4 matrices studied when compared with 3 other protocols used in this study. Nonetheless, this protocol allowed for more rapid results. Therefore, because there were no differences among the isolation abilities of these 4 protocols in inoculated Butterfield's solution and poultry litter, it is anticipated that

[11]Product No. Z5342, Promega Inc., Madison, WI.
[12]SAS User's Guide, Version 8.0, SAS Institute Inc., Cary, NC.

TABLE 1. Minimum concentration of *Salmonella* detected in 4 different spiked matrices with 4 different isolation protocols

Protocol	Sample matrix (log_{10} cfu/mL)			
	Pure culture	Litter	Crop	Carcass rinse
Dynabeads	3.66 ± 1.51[a]	2.09 ± 0.79[a]	3.97 ± 1.43[a]	3.06 ± 0.63[a]
Rappaport-Vassiliadis	2.96 ± 0.48[a]	1.89 ± 0.52[a]	2.17 ± 0.42[b]	0.96 ± 0.48[b]
Tetrathionate	3.46 ± 0.92[a]	1.99 ± 0.48[a]	2.47 ± 0.32[b]	0.86 ± 0.52[b]
Secondary enrichment	2.56 ± 1.26[a]	1.79 ± 0.53[a]	2.07 ± 0.48[b]	0.76 ± 0.53[b]

[a,b]Mean values (n = 10) within a column with the same superscript are not significantly different ($P > 0.05$) using Tukey's adjustment of least square means.

DB may be useful when certain matrices are tested under a preharvest *Salmonella* monitoring regimen.

The reduced efficacy of *Salmonella* isolation by DB from spiked crop and carcass rinse samples may well be directly related to the composition of these matrices. Previously, in using IMS in various food products, it was determined that a sample matrix could affect the isolation ability of a method (Skjerve and Olsvik, 1991).

The crops consist of muscle and fat, the components of which are proteins and lipids. Any of the components of the crops, alone or in combination with other constituents, could provide a physical or chemical barrier that could interfere with the binding sites on the anti-*Salmonella* antibodies attached to the magnetic beads. Another factor to consider is that direct physical attachment of bacteria to the components of the matrix could be stronger than the attraction to the antibodies on the magnetic beads in this context. Similarly, for the carcass rinse samples, fatty components that were washed from the carcass during the rinse process may contribute to decreased detection for the DB protocol. The use of cheesecloth has been used to remove such inhibitory components when evaluating IMS in carcass rinse samples (Wang and Slavik, 1999). In the current study, no sample filtration techniques were used because they can potentially remove *Salmonella* that may be present within the sample. Wang and Slavik demonstrated an average isolation rate of 4.36 log_{10} cfu/mL with filtration, and in the current study the DB average isolation rate was 3.06 log_{10} cfu/mL without the use of cheesecloth.

The use of delayed secondary enrichment (DSE) has been shown to dramatically increase the *Salmonella* isolation from various sample matrices (Pourciau and Springer, 1978; Waltman et al., 1991; Nietfeld et al., 1998). Most DSE protocols require extended incubation of primary enrichment samples from 5 to 10 d at ambient temperatures if no suspect colonies are present after plating the initial enrichment. After primary enrichment for 24 h, samples are left at room temperature for an extended time, and an aliquot is subsequently transferred to fresh selective enrichment broth and further incubated at elevated temperatures overnight. Samples are then replated onto selective agar plates. A shortened modification of the DSE method was used as the fourth *Salmonella* isolation method for this study.

This method, secondary enrichment, used TT as the primary enrichment broth and RV as the secondary enrichment. Also, the original TT broth was incubated only for 48 h at elevated temperatures as opposed to 5 to 10 d at ambient temperatures as common DSE methodology does. The TT, which contains calcium carbonate, provides an optimal environment for the *Salmonella* to proliferate, but at the same time other microorganisms that are present may grow. Therefore, the use of RV, which contains malachite green, a substance that is toxic to many bacterial species, eliminates the competing organisms when this secondary enrichment is used.

In this work, there were no statistically significant differences in the isolation abilities of the 4 protocols in 2 of the 4 matrices (PC and litter) studied. However, the shorter time required to obtain results when using the DB method could provide an advantage in certain matrices from a commercial operation. On the other hand, the DB method may not be as effective as the other methods in samples from chicken tissues. Therefore, further work is warranted to determine whether the DB isolation protocol would provide the same advantage observed in this study when examining actual field samples contaminated with naturally occurring levels of *Salmonella*.

It should be noted here that the cost of the various *Salmonella* isolation methodologies used is varied. The DB is considerably more expensive than the other 3 methods used. However, if the DB method can reduce the analysis time by 1 to 3 d and proves to be at least as sensitive as the other methods, it could be worth the additional cost in a production and process risk-assessment scenario.

This work demonstrates that during microbial risk assessment, attention should be given to the type of matrix that the *Salmonella* is to be isolated from as well as the microbiological isolation methods used. It is essential that risk-management decisions be based on well-defined and characterized risk-assessment methods.

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